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Prevalence of *Mannheimia haemolytica* Isolated from Bovine Nasal Exudates and Associated Factors, in Dairy Farms in the North-Central of Mexico

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Abstract: *Mannheimia* sp. strains obtained from bovine nasal exudates of either clinically healthy (n = 1902) or infected with pneumonia animals (n = 189) were isolated and characterised to estimate the prevalence of isolated serotypes and to identify some factors associated to prevalence in dairy farms in Mexico, by means of a transectional descriptive study. Strains were isolated and typified through conventional *in vitro* culture methods, biochemical and immunological tests. Chi square or fisher statistical tests were applied, as well as odds ratio calculation and logistic regression analysis to evaluate the association and effect of some variables on *Mannheimia* sp. isolation. Isolates were composed in 48% by serotype A1, in 2.4% by A6 and 49.3% were non-typable. The apparent prevalence rates of *Mannheimia haemolytica* were significantly higher in diseased bovines (OR = 2.54; $p<0.05$), as well as in bovines younger than 1 year of age (OR = 2.26; $p<0.05$). Health condition and age were the variables that remained in the logistic regression model. Serotype A1 showed the highest prevalence, even when most isolates were not-typable. Bovines younger than one year of age and those with respiratory disease were the groups with the highest frequency of *M. haemolytica* isolates.

Key words: *Mannheimia* serotypes, bovine mannheimiosis, prevalence, Mexico

INTRODUCTION

Pasteurella haemolytica A serotypes were reclassified into new genus *Mannheimia* and the A serotypes of *P. haemolytica* (A1, A2, A5-A9, A12-A14, A16 and A17) were renamed *M. haemolytica* (*Mh*). Serotype A11 was reclassified into the new species *M. glucosida* (Mg) (Angen *et al*., 1999a, b).

Mh is present in the nasopharynx and tonsils of apparently healthy animals (Rowe *et al*., 2001), this microorganism is the one most frequently associated to bovine pneumonic pasteurellosis, called shipping fever pneumonia, affecting mainly animals younger than one year of age, recently transported or added to the herd during the first year of live (Murphy *et al*., 1993; Trigo, 1991). It is also considered the disease most relevant in terms of economic aspects in the bovine industry worldwide (Narayanan *et al*., 2002).

The predominant serotype recovered from healthy calves or pneumonic lesions in cases of shipping fever is Mh A1, though serotypes A2, A5, A6, A7, A9, A11, A12 and A14 are sometimes recovered (Quirie *et al*., 1986; Wray and Thompson, 1971).

Studies performed in Mexico since the 1980s report that A1 and A2 are the most frequent serotypes in pneumonic lungs or in clinically healthy bovines (Blanco *et al*., 1995; Pijoan *et al*., 1999; Sánchez *et al*., 1988).

Considering that Mh is the main bacterial pathogen of bovine pneumonic pasteurelosis (Narayanan *et al*., 2002; Al-Ghamdi *et al*., 2000) for the purpose of this study, we will refer to the latter as mannheimiosis along the text.

In Mexico, there has been no overall epidemiological evaluation of ruminant pneumonia and no reliable information is available on the prevalence and characterisation of the main bacterial species responsible for pneumonia in bovines (Pijoan *et al*., 1999). In addition, available information on incidence and prevalence in bovines is hardly reliable and very scarce and unclear, which renders its analysis very difficult.

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The objective of the present study was to isolate the strains of *Mannheimia* spp from nasal exudates of clinically Healthy pneumonia-free (H) and clinically Diseased with pneumonia (D) bovines and to determine the prevalence of the different isolated serotypes and some factors associated with their prevalence in 320 dairy farms located in the Comarca Lagunera in the States of Coahuila and Durango, Mexico.

MATERIALS AND METHODS

Study area: The Comarca Lagunera is a semidesert region in North-Central Mexico located at 24° 22' N, 102° 22' W, constituted by 11 counties in the State of Durango and 5 counties in the State of Coahuila (INEGI, 2006). It is the most important dairy region of Mexico with 2,081,000 litres of milk production annually, representing 20% of the domestic milk production and a dairy cattle population of approximately to 440,876 (SAGARPA, 2006).

Sampling design: The Comarca Lagunera has 320 Dairy Farms (DF) and an average of 2500 bovines per DF. With a sample frame constituted by 216 DF, a random two-stage sampling was used according to the method proposed by Segura and Honhold (2000). During the first stage, to obtain an estimated prevalence value to calculate sample size, a simple random pilot sampling was performed on 10% of the DF (more than 10% of non-responsive) (n = 24 DF).

With the estimated prevalence values obtained in the pilot sampling, we calculated the parameters required to estimate the minimum sample size per conglomerate (dairy farm) of random sampling, with the following equation (Segura and Honhold, 2000).

$$n = \frac{t^2 s_b^2}{d^2} = 1707.6$$

where t is the critical value of the Student's t table, with an infinite number of degrees of freedom (confidence level of 95%), S_b^2 is the total variance that include the variance among and within conglomerates and d is the desired precision (3%).

The number of DF (n = 36) to be sampled was obtained by dividing the total sample size (1707.6) between the mean of animals sampled within each DF (48) and in a second stage, it was decided to sample 20 DF, which added to the 24 of the first stage gave a total of 44 DF. Samples of 2% of the H-bovines were obtained from each of the 44 DF (n = 1902), always verifying that 70% were less than 1 year old, because of the greater susceptibility of this age group to pneumonic problems. Additionally, samples were obtained from 100% of the D-bovines (n = 189).

Sampling: We considered animals as clinically Diseased with pneumonia (D) those presenting clinical manifestations of an undifferentiated respiratory disease, such as: Nasal discharge, coughing, hyperpnoea or dyspnoea, pyrexia and retarded growth. Those animals not presenting these clinical manifestations were considered clinically Healthy pneumonia-free (H).

Samples were obtained from the deep nasal cavity of H-(n = 1902) and D-bovines (n = 189) by rotation of a sterile cotton swab (Copan Venturi Transystem, Copan, Italy) with Amies medium and activated charcoal. These were kept under refrigeration for no more than 24 h until processed at the bacteriology laboratory of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP).

Strain isolation and identification: Cotton swabs were cultured by inoculation in blood agar and 5% sheep blood (BBL, Becton Dickinson) and incubated at 37°C for 24 h. Phenotype identification of colonies with typical appearance of *Mannheimia sp.* was done by conventional methods of gram-staining and biochemical tests (oxidase, carbohydrate fermentation and sulfhydric acid production (TSI), citrate utilisation, motility and indole production, urease production, trehalose and aesculine fermentation). A single representative colony was chosen for evaluation. These colonies were sub-cultured in blood agar (5% sheep blood) (37°C/24 h) and pure cultures of the strain were obtained. Final identification was done with the API 20E bacterial identification system (bioMerieux, Durham, NC, USA. Inc). Briefly, cultures were resuspended in 5 mL of a sterile suspension medium at a density of 4.0 on the McFarland scale. The suspension was transferred to the biochemical test strips and reactions were read after incubation at 37°C for 24 h, according to manufacturer's instructions. All stated biochemical tests were also applied to reference strains of Mh (Strains 1, 2, 5-9, 11, 12) and *P. trehalosi* (Strains 3, 4, 10) (kindly donated by Dr. GH Frank and Dr. B. Briggs, NADC, USDA).

Results of the profile of each strain were compared with the profiles of the taxa in the data base contained in the Program APIWEB (http://www.biomerieux.com). In addition, the phenotypical properties of *Mannheimia sp.* described by other authors (Angen *et al*., 1999a, 2002) were taken into account (Table 1).

According to the threshold values proposed by the API 20E system, it was considered that strains with an identification percentage (%ID) under 80% had an Unacceptable Profile (UP), with a %ID above 80% they belonged to the same genus (*Mannheimia* sp.) and with a %ID above 90% to the same species (Mh).

Table 1: Phenotypic reactions characterising the different species in the *Mannheimia* genus*

Test	*M. haemolytica*	*M. glucosida*	*M. varigena*	*M. granulomatis*	*M. ruminalis*
Mannitol	+	+	+	+	+
Maltose	+	+	+	+	+/-
D-Xilose	+	+	+	+/-	+/-
D-Sorbitol	+	+	-	+	+/-
α-fucosidase (ONPF)	+	+	+/-	-	-
Haemolysis	+	+	+	-	-
ß-galactosidase (ONPG)	+/-	+	+/-	+/-	+
ß-glucosidase (NPG)	-	+	+/-	+	-
Glycosides[a]	-	+	-	+/-	-
L-Arabinose	-	+/-	+	-	-
Ornithine decarboxylase	-	+/-	+/-	-	-
Indole	-	-	+/-	-	-
Urease	-	-	-	-	-
Trehalose	-	-	-	-	-

*Angen *et al.*, 1999a; Angen *et al.*, 2002, +: Positive ; -: Negative; +/-: Positive or negative, [a] Amygdalin, aesculin, arbutin, cellobiose, gentobiose

Serologic typification: This was performed by the IHA technique described by Biberstein (1978) and monospecific antisera against capsular antigens (1-17) of *Mannheimia* sp. were used (kindly donated by Dr. GH Frank and Dr. B. Briggs, NADC, USDA). Agglutinations with titres above 1: 64 were considered positive. Each plate included a positive and a negative control.

Identification of factors associated to *Mannheimia* sp. prevalence: A questionnaire was designed and applied at each of the sampled DPU (n = 44) with the purpose of identifying some variables probably associated to the epidemiology of mannheimiosis, among which were included: animal age, calf accommodations, vaccines applied to prevent diseases of the respiratory tract (infectious bovine rhinotracheitis, parainfluenza 3, bovine syncytial virus and mannheimiosis), as well as to determine the degree of association with *Mannheimia* sp. prevalence.

Statistical analysis: Statistical analysis was done with the Epi Info®, Version 3.3.2 program (Centers for Disease Control and Prevention, Atlanta, GA, USA, 2004). Data were used to calculate prevalence rates. In addition, the Chi square or Fisher tests were applied depending on the data characteristics to evaluate associations and OR were calculated to determine the degree of association. A logistic regression analysis was applied to evaluate the joined effect of the variables, in which the dependent variable was isolation or no isolation of *Mannheimia* sp. Only variables with a $\leq$0.1 probability of error obtained by univariate analysis with Chi square were selected and those with p$\leq$0.05 to remain in the model.

RESULTS

Strain isolation and typification: All isolates (n =127) were gram-negative, haemolytic, non-mobile coccobacilli, positive to cytochrome-oxidase production, to nitrate and nitrite reduction and to D-glucose, D-sucrose and D-mannitol fermentation and negative to the following tests: Arginine dehydrolase, lysine decarboxylase, ornitine decarboxylase, citrate utilization, sulfhydric acid production, tryptophan deaminase, urease and indole gelatinase production, trehalose, L-rhamnose, D-melobiose, L-arabinose fermentation and McConkey agar growth. Variations were found in the percentages of isolates positive to some tests (Table 2).

According to the threshold values established by the API 20E system, of the 127 analysed strains, 126 (99.2%) showed a %ID above 90% and thus corresponded to Mh and 1 (0.78%) showed a %ID above 80%, corresponding to *Mannheimia* sp. genus.

Serotyping: Of the 127 isolates, 61 strains (48%) were serotype A1, 3 (2.4%) were serotype A6 and 63 strains (50.1%) were Non-Typable (NT). All strains identified as A1 and A6 and 62 NT showed phenotypic properties with %ID above 90% and 1 strain NT showed phenotypic properties with %ID above 80% according to other authors (Angen *et al.*, 1999a, 2002). In the H-animals 51.4% were A1; 2.9%, A6 and 46%, NT; in D-animals, 33% were A1; and 67%, NT. The differences in the frequency among serotype isolates in H-and D-bovines were significant (p = 0.05) with the Chi square test.

Isolate prevalence rates: In H-bovines (n = 1902), 103 isolates were obtained; in D-bovines (n = 189), 24 isolates were obtained. In H-bovines, the apparent prevalence rate (APR) of Mh was 5.4% ($CI_{95\%}$ 4.3% to 6.4%), in D-bovines it was 12.7% ($CI_{95\%}$ 7.9% to 17.4%). The differences between TPA in H-and D-bovines were significant (r = 2.2, $CI_{95\%}$ 1.24 to 3.23) with the independent rates analysis.

Associated factors: Among the associated factors, statistical significance in the frequency of Mh isolations was only found in relation with health conditions and age group (Table 3). Mh isolation frequency was higher in: A) D-bovines than in H-bovines (OR = 2.54 [1.54-4.16])

Table 2: Phenotypic characteristics of *Mannheimia* sp. strains

Phenotypic characteristics	Positive (%)
Haemolysis	100
Cytocrhome oxidase	100
Nitrate reduction	100
D-glucose	100
D-sucrose	100
D-mannitol	100
ß-galactosidase (ONPG)	100
Inositol	39.4
D-sorbitol	32.3
Amygdalin	0.78
Aesculin	0
Motility	0
Voges Proskauer	0
MacConkey medium growth	0
Gelatinase	0
Lysine descarboxylase	0
Arginine dehydrolase	0
Ornithine Decarboxylase (ODC)	0
Citrate	0
Indole	0
H_2S	0
Urease	0
Tryptophane deaminase	0
Trehalose	0
L-rhammnose	0
D-melobiose	0
L-arabinose	0

Table 3: Factors associated with *M. haemolytica* isolates in bovines from dairy farms in the North-Central of Mexico

Associated factors	OR (CI 95%)	P
Diseased vs. healthy	2.5 (1.5-4.1)	<0.001
Age under 1 vs. over 1	2.2 (1.4-3.8)	<0.001
Outdoors vs. indoors	1.7 (0.6-4.9)	0.242
Individual vs. group	2.5 (0.7-9.9)	0.170
Vaccinated Yes vs. No	2.3 (0.5-14.7)	0.161

OR, Odds ratio and confidence interval 95%; p, significant difference, (p = 0.05) or not significant (p> 0.05) to Chi square or Fisher tests

Table 4: Logistic regression model for *M. haemolytica* isolation in bovines from dairy farms in the North-Central of Mexico

Risk factor	α	α	OR	CI 95%	P
Age[a]	-3.45	0.77	2.17	1.33 – 3.53	< 0.001
Health conditions[b]	-3.45	0.87	2.39	1.49 - 3.85	<0.001

[a]Age under 1 vs. over 1, [b] Diseased vs. Healthy

(p<0.05) and b) in bovines younger than one year than in older animals (OR = 2.26 [1.36 – 3.8]) (p<0.05). The variables of age and health condition were included in the logistic regression analysis and both of them remained in the model (Table 4).

DISCUSSION

Aimed at obtaining a more reliable phenotypic characterization of the isolates, we performed conventional biochemical tests aside from the API 20 system to provide support to the serotypification, considering that some authors claim that serotyping by IHA alone is not sufficiently specific for reliable identification of *M. haemolytica*, if not supported by several biochemical tests, since the genus *Mannheimia* includes phenotypically and genotypically very heterogeneous taxa (Angen *et al*., 1999a, 2002).

No other bovine nasal exudate studies have been performed in Mexico, the highest prevalence of serotype A1 among serotypable strains in the H- (51.4%) and D- (33%) bovine groups agrees with reports from other authors, who describe this serotype as the most frequent in bovines (Rowe *et al*., 2001; Wary and Thompson, 1971).

Prevalence in the H-bovines was similar to the 50% found in nasal exudates of healthy calves by Wray and Thompson 1971) in Great Britain. Frank (1982) showed that the A1 serotype predominates in calves transported to auction barns and feed-yards and Rowe *et al*. (2001) have also reported serotype A1 as the most frequent in tracheobronchial washings of bovines.

The present is the first isolation of the A6 serotype from bovine nasal exudates in Mexico, since A6 has only been reported in ovine and bovine lungs (Blanco *et al*., 1995, 1993). In other countries, serotype A6 has been reported in ovine lungs in Great Britain (Fraser *et al*., 1982a), Ethiopia (Sisay and Zerihun, 2003), Turkey (Kirkan and Kaya, 2005) and in bovine lungs in the United States of America (Al-Ghamdi *et al*., 2000).

In the present study, the frequency of NT strains, in both H-(46%) and D-(67%) animals is higher than the frequencies found by other national and international authors. Frequencies of 14.6% NT strains have been reported in nasal exudates of ovines in Mexico (Argueta *et al*., 1988) and 9.3% of NT strains have been reported in calves in Great Britain (Wray and Thompson, 1971).

Frequencies of NT strain isolates are very variable and can sometimes be high (Fraser *et al*., 1982b) and differ depending on the source of isolation: Great Britain reports 24% in pneumonic calves lungs (Quirie *et al*., 1986) Denmark, 25% (Angen *et al*., 2002) United States, 15.8% in sheep nasal exudates (Frank, 1982); Ethiopia, 3.6% (Sisay and Zerihun, 2003), Hungary, 7% (Fodor and Varga, 1998); and Turkey, 8.3% (Kirkan and Kaya, 2005) in ovine lungs. These NT strains normally correspond to the A biotype (Frank, 1980) and have been described as Mh mutants, some of which are deficient in soluble antigen production (Gentry *et al*., 1988).

It has been stated that isolates frequency is low in nasal cotton swabs of healthy, non-stressed animals and high in calf with respiratory tract disease (Frank and Smith, 1983). This agrees with the prevalence values found in this study, which were higher in D- animals.

Information on frequency, prevalence and incidence is scarce and unclear in Mexican studies. In calves, incidence ranges from 19.7 to 39.9% (Pijoan and Chávez, 2003), or frequency from 29 to 49% of *P. haemolytica* serotypes in sera (Sánchez *et al*., 1998), 12% *P. haemolytica* isolates in ovine nasal exudates

(Argueta *et al*., 1988) and 25 to 35% in ovine or calf lungs (Blanco *et al*., 1995, 1993; Pijoan *et al*., 1995). These studies were performed with intentional samples mostly obtained at diagnostic centres, slaughterhouses and to a lesser degree at livestock units. They are not sufficiently representative of reference populations, neither in quality nor in quantity, so that rates do not allow valid inferences concerning these populations.

In the present study, random sampling of the reference population and the isolate frequencies obtained constitute the APR, which allowed to infer, with a 95% confidence index, the actual prevalence rates (RPR). In H-bovines, RPR values for Mh range from 4.3 to 6.4% and in D-bovines, they range from 7.9 to 17.4%.

The present results are only partially comparable with other national and international studies, since the samples used in other studies are different in origin and characteristics. In H-bovines, the Mh APR (5.4%) obtained was lower than that found in calf nasal exudates by Frank and Smith (1983) (17%), Wray and Thompson (1971) (87.7%) and that reported by Argueta *et al.* (1988) (12%) in ovine nasal exudates. In D-bovines, Mh APR (12.7%) was lower than the 25% frequency reported by Zanabria *et al.* (2000) in bovine nasal exudates and similar to that found by Sisay and Zerihun (2003) in ovine nasal exudates (13%).

No previous studies have been published in Mexico concerning the evaluation of Mh and its distribution pattern with respect to predisposing factors, such as age, health conditions, or other determining factors. Most studies, national and international, concentrate on the clinical aspects and pathogenicity of the *Mannheimia* genus.

The higher frequency of Mh isolates in D- than in H-bovines (OR = 2.54 [1.54-4.16]) and in bovines under one year of age than in older animals (OR= 2.26 [1.36-3.8]), confirms that isolates of the genus *Mannheimia* are more frequent in young, especially under one year of age and diseased animals, because they are consistently the most exposed groups. This agrees with statements by other authors (Wray and Thompson, 1971).

Differences in *Mh* isolation frequency according to the type of calf accommodations were in no case significant. There are doubtlessly other confounding variables that intervene such as: Management, hygiene, colostrum quality and supply and weather conditions, which should be evaluated in more detail. The influence of the type of accommodations in the presence of pneumonia is controversial (Pijoan and Chavez, 2003). It has been reported that there are no significant differences if calves are indoors or outdoors regarding health (Jorgenson *et al*., 1970) and no association between health and cage dimensions or materials have been found (Fisher *et al*., 1985). All other analysed variables showed no significant differences or consistency to allow comparisons or solid conclusions.

These results allow us to conclude that the isolates obtained in this study, together with those of another study we are currently performing in a different region of Mexico (unpublished results), constitute the most representative collection of *Mannheimia* strains of bovine origin in the country. Most isolates were NT, followed by the A1 serotype as the most frequent; confirming other reports (Quirie *et al*., 1986; Frank, 1982). According to the APR, bovines younger than one year of age and those with pneumonic disease were the groups with the highest frequency of Mh isolates.

Further studies are underway with molecular biology techniques for definitive identification of the isolated strains.

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